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Depletion of tyrosyl-DNA phosphodiesterase 1α (*MtTdp1α*) affects transposon expression in *Medicago truncatula*

Summary The role of plant tyrosyl-DNA phosphodiesterase 1α in genome stability is studied using a *Medicago truncatula MtTdp1α*-depleted line. Lack of *MtTdp1α* results in a 39% reduction of methylated cytosines as compared to control. RNA-Seq analyses revealed that 11 DNA transposons and 22 retrotransposons were differentially expressed in the Tdp1α-2a line. Among them all, DNA transposons (*MuDR*, *hAT*, *DNA3-11_Mad*) and seven retrotransposons (LTR (Long Terminal Repeat)/*Gipsy*, LTR/*Copia*, LTR and NonLTR/L1) were down-regulated, while the 15 retrotransposons were up regulated. Results suggest that the occurrence of stress-responsive *cis*-elements as well as changes in the methylation pattern at the LTR promoters might be responsible for the enhanced retrotransposon transcription.

MtTdp1α (tyrosyl-DNA phosphodiesterase) gene depletion affects *Medicago truncatula* genome stability, as reported in the present work. The *MtTdp1α*-depleted line Tdp1α-2a showed 39% reduction in overall methylated cytosines, compared to the control (CTRL) line. According to RNA-Seq, 33 transcripts resulting from 11 DNA transposons and 22 retrotransposons, respectively, were differentially expressed in the Tdp1α-2a line. All DNA transposons (*MuDR*, *hAT*, *DNA3-11_Mad*) and eight retrotransposons, namely LTR/*Gipsy*, LTR/*Copia*, LTR and NonLTR/L1, were down-regulated. The remaining LTR/*Gipsy*, LTR/*Copia*, LTR and NonLTR/SINE (Short Interspersed Nuclear Element) retrotransposons were up-regulated. Reduced methylation levels were observed at the GYPSY-LTR_MT sequence hereby analyzed.

Transposable elements (TEs) excision causes Double Strand Breaks (DSBs) which activate DNA damage sensing/signaling mechanisms; however, the choice of the proper repair pathway for the removal of transposition-mediated DSBs is dependent on host-specific repair factors and cell cycle phase (Izsvak et al. 2009). In a recent report, Dinh et al. (2014) demonstrated that the *Arabidopsis thaliana* DNA topoisomerase (topo) Iα participates in the transcriptional silencing of TEs since this function was required to inhibit RNA-directed DNA methylation, one of the main factors controlling TEs silencing. DNA topoisomerases are key regulators of DNA topology in the early steps of DNA repair (Balestrazzi et al. 2010). In the presence of damaged DNA sites or topo I drugs, the transient covalent complexes topo I/DNA formed during the catalytic cycle are stabilized, resulting in cytotoxic DSBs. Tyrosyl-DNA phosphodiesterase I (Tdp1) enzyme is able to break the covalent linkage between the DNA termini and the catalytic tyrosine residue of topo I, thus releasing the enzyme trapped on the double helix (Interthal et al. 2001). The *Tdp1* gene family from *Medicago truncatula* Gaertn., including *MtTdp1α* and *MtTdp1β* genes, has been characterized (Macovei et al. 2010). RNA-Seq analysis, carried out in the *MtTdp1α*-depleted *M. truncatula* plants, has identified gened differentially expressed that are involved in DNA damage sensing, DNA repair and chromatin remodelling (Donà et al. 2013b).

There is still limited information on TEs mobilization profiles occurring *in planta* in presence of defective DNA repair. In this work, the molecular characterization of the *M. truncatula MtTdp1α*-depleted line Tdp1α-2a (Donà et al. 2013b) has been expanded, assessing the global cytosine methylation profile, TEs expression patterns, occurrence of TEs-associated stress-responsive *cis*-regulatory elements and possible changes in the methylation patterns at their promoter regions. In our opinion, the reported data add novel information concerning the role of Tdp1 in relation to genome stability issues in plants, expanding the current knowledge on a crucial, still uncovered, molecular aspect of plant physiology.

In the present work, the link between the lack of *MtTdp1α* gene function and TEs transcriptional profiles has been investigated in the model legume *M. truncatula*, in order to provide novel insights into the role played by the DNA repair enzyme Tdp1α in safeguarding the plant genome. The RNA-Seq database used in the present work has been produced in a previous study (Donà et al. 2013b). The sequencing data used in this work are deposited to the NCBI Short Read Archive and are accessible through SRA accession number SRP013555 (http://www.ncbi.nlm.nih.gov/Traces/sra/sra.cgi). Gene sequences were obtained from http://medicagohapmap.org based on genome release Mt3.5. Amino acid sequences were compared using the Expasy SIB BLAST Network Service (http://www.expasy.ch/tools/blast//). TopHat software (tophat.cbcb.umd.edu) was used for data assembly. Data available from RNA-Seq were assembled using the TopHat software (tophat.cbcb.umd.edu) and subsequently analyzed using the Cufflinks v.1.3.0 (Trapnell et al. 2010) program for the identification of unannotated transcripts. As a result, 1,480 putative new transcripts were identified, among which 150 showed differential expression in the Tdp1α-2a line compared to CTRL line. BLAST alignments revealed that 33 out of the 150 differentially expressed genes contained TE-related sequences. A detailed list of the 33 sequences is provided in Tables S1, S2 and S3. Each sequence was classified using the RepBase alignment tool (www.girinst.org/repbase/index.html). Eleven sequences were DNA transposons while the remaining 22 sequences were retrotransposons (Figure 1A). DNA transposons were assigned to *MuDR*, the autonomous element of the *Mutator* transposon family and to *hAT(Hatvine)* DNA transposon superfamily (Table S1). The 22 differentially expressed retrotransposons, were assigned to nine different groups (Tables S2, S3) which

include LTR retroelements belonging to the main plant superfamilies, *Gypsy* and *Copia*, and non-LTR retrotransposons with LINE (Long Interspersed Element) and SINE components (Table S3).

Since it is known that in yeast the *Tdp1* gene is required to ensure the accurate processing of DNA ends during non-homologous end joining (NHEJ) (Bahmed et al. 2010), it is possible that the impairment of DNA transposon mobility in the *MtTdp1α*-depleted cells might be caused by defective NHEJ. Although no significant differences were evidenced by RNA-Seq analysis in the expression profiles of *M. truncatula* genes involved in the NHEJ pathway (Donà et al. 2013b), the occurrence of post-transcriptional regulation of NHEJ proteins cannot be ruled out. To date, no direct evidence of the involvement of Tdp1 enzyme in the resolution of specific DNA structures that mediate TE transposition is available. However Heo et al. (2015) recently demonstrated that hTdp1 physically interacts with NHEJ proteins recruited at DSBs sites during the early step of this repair. The overall perturbations of the DNA repair response affecting Nucleotide Excision Repair (NER) and Base Excision Repair (BER) pathways might be partially responsible for the enhanced mobilization of LTR and non-LTR retrotransposon. It is known that the yeast RAD27/FEN(FLAP ENDONUCLEASE) 1 nuclease activated during the BER process, is able to affect the stability of complementary DNA (cDNA) intermediates produced during the retrotransposition of *Ty1* element (Sundararajan et al. 2003). In animal cells, the NER component ERCC(EXCISION REPAIR CROSS-COMPLEMENTATION GROUP) 1/XPF(XERODERMA PIGMENTOSUM GROUP F) is required to limit the mobility of the non-LTR retrotransposon LINE-1 (Gasior and Deininger 2008). The involvement of NHEJ pathway in LINE-1 retrotransposition is currently discussed since in animal cells with defective NHEJ, a decrease in mobility of LINE-1 has been demonstrated (Suzuki et al. 2009).

All DNA transposons were down-regulated at the transcriptional level in the Tdp1α-2a line while 15 out of the 22 retrotransposons were up-regulated (Figure 1B). LTRs, accounting for 20 out of the 22 retrotransposons retrieved, include 14 out of the 15 up-regulated TEs. To find out factors responsible for enhanced retrotransposon transcription, the promoter region of each LTR retroelement (LTR/*Gipsy*, LTR/*Copia*, LTR; Table S2) was retrieved and analyszed for putative *cis*-acting regulatory elements. The search for putative *cis*-acting regulatory elements was performed using the PlantCARE database (bioinformatics.psb.ugent.be/webtools/plantcare/html/). The graphical representation of *cis*-element abundance in the promoter region of the differentially expressed LTR retroelements is shown in Figure 1C, where data are expressed as percentage of occurrence of a single hit on the total number of 69 *cis*-elements identified. The most abundant *cis*-elements include TGACG and CGTCA (methyl-jasmonate responsive), TC-rich (defense and stress responsive), and ARE (Anaerobic Responsive) elements (7/69, 10.14%), followed by HSE (Heat Stress Responsive) (6/69, 8.69%). GC (anoxic specific inducibility enhancer), TCA (salycilic acid responsive) and ABRE (ABA responsive) motifs were less frequent elements (5/69, 7.24%). Other motifs related to pathogen interaction were poorly represented (Figure 1C). The LTR promoters harboring stress-responsive regulatory elements might undergo constitutive activation in the *MtTdp1α*-depleted cells, possibly contributing to TE-mediated genome destabilitation.

It has been reported that TE may contain *cis*-regulatory elements that act as binding sites for stress-inducible transcription factors. Indeed, Makarevitch et al. (2015) found the DRE (Dehydration Responsive Element) consensus sequence in most of the TEs associated with stress-responsive genes in maize. The stress-related *cis*-regulatory elements were found in the main TE families, including LTR/*Gipsy*, LTR/*Copia* and LINE, spread uniformly across the maize genome (Makarevitch et al. 2015). Stress-responsive *cis*-elements were also detected in the retrotransposons activated in the *MtTdp1α*-depleted cells. It is possible that these regulatory elements might have contributed to enhance the expression of the stress-responsive genes, as highlighted by RNA-Seq (Donà et al. 2013b).

All the reported analyses were carried out using 20-d-old *M. truncatula* Gaertn. (cv. Jemalong, M9-10a genotype) plants (Tdp1α-2a and CTRL lines) grown *in vitro* as described by Donà et al. (2013b). The global 5-methylcytosine (5-mC) levels were assessed in genomic DNA extracted from leaves using the 5-mC DNA ELISA kit D5325 (Zymo Research Corporation, Irvine, California, U.S.A.) according to supplier's indications. DNA aliquots (100 ng) were denaturated and incubated with a mix consisting of anti-5-methylcytosine and secondary (horseradish peroxidase conjugate) antibodies. For each DNA sample, the percentage of 5-mC was quantified by plotting absorbance versus 5-mC percentage in the standard curve. For each *M. truncatula* line, duplicated DNA samples for two independent replications were analyzed. As shown in Figure 1D, a significant ($P < 0.001$) reduction of $39.0 \pm 2.0\%$ in the 5-mC amount was observed in the *MtTdp1α*-depleted line, compared to CTRL line.

The global loss of methylated cytosine residues detected in the Tdp1α-2a line and the loss of methylation observed at one of the LTR sequences that were transcriptionally up-regulated are in agreement with the expression profiles of key genes involved in DNA methylation provided by RNA-Seq analysis (Donà et al. 2013b). Thus, the observed DNA hypomethylation might have contributed to retrotransposon deregulation. Although this investigation does not provide direct evidence of TE excision, it is acknowledged that transcription is the first step of retrotransposition (Casacuberta and Santiago 2003); however, in-depth studies will be necessary to disclose this specific aspect. Changes in the methylation patterns at the LTR promoter regions might be responsible for the enhanced retrotransposon transcription. In order to assess the methylation state at LTR promoters, genomic DNA digestion with methylation-sensitive endonucleases followed by PCR-mediated amplification was carried out on a single LTR sequence (GYPSY-LTR_MT, Table S2, TCONS_00084148) selected as representative of the differentially expressed LTR retrotransposons previously described. As shown in Supplemental Figure S1, in the CTRL line the target LTR region was not cleaved by the methylation-sensitive endonucleases *Hpa*II and *Sac*I, due to the presence of methylated cytosine residues, while the low amplification efficiency observed with the DNA from Tdp1α-2a line possibly resulted from the *Hpa*II- and *Sac*I-mediated cleavage at demethylated LTR sites.

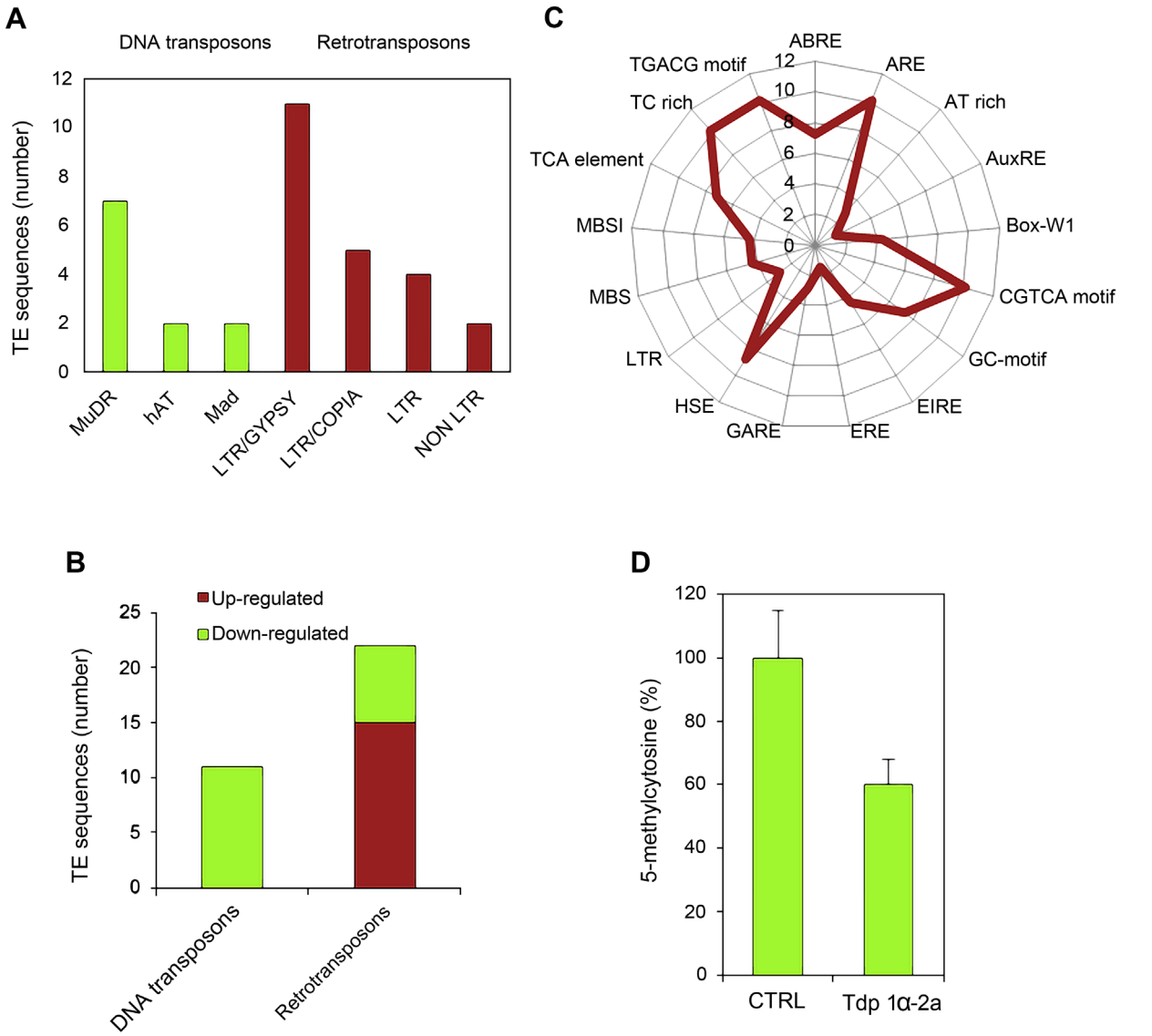

Figure 1. Effects of *MtTdp1α* gene depletion on DNA transposon and retrotransposon expression and global cytosine methylation

(**A**) Transposable elements (TEs)-related gene sequences differentially expressed in the *M. truncatula* *MtTdp1α*-depleted line Tdp1α-2a compared to control (CTRL) line included two major classes, DNA transposons and retrotransposons. A more accurate classification was obtained using the RepBase alignment tool (www.girinst.org/repbase/index.html). (**B**) Differential regulation of TEs in the *MtTdp1α*-depleted line Tdp1α-2a as highlighted by RNA-Seq analysis. All the DNA transposons were down-regulated while most of the retrotransposon (15 out of 22) were up-regulated. *MuDR*, autonomous element of the *Mutator* transposon family. LTR, Long Terminal Repeat. (**C**) Radar chart of *cis*-element distribution in the promoter regions of the LTR retroelements differentially expressed in the *MtTdp1α*-depleted line Tdp1α-2a, compared to CRTL line. Data are expressed as percentage of occurence of a single hit on the total number of recorded *cis*-element. ABRE, ABA Responsive Element. ARE, Anaerobic Responsive Element. AT-rich, ATBP-1 Binding site. AuxRE, Auxin Responsive Element. Box-W1, Responsive to Fungal Elicitor. CGTCA motif, Methyl-Jasmonate (MeJA) Responsive element. GC-motif, Anoxic Specific Inducibility Enhancer. EIRE, Elicitor Responsive Element. ERE, Ethylene Responsive Element. GARE, Gibberellin Responsive Element. HSE, Heat Stress Responsive Element. LTR, Low Temperature Responsive. MBS and MBSI, Myb-Binding Site. TCA-element, Salicylic Acid (SA) Responsive Element. TC-rich, Defense and stress responsive. TGACG motif, MeJA Responsive Element. (**D**) Quantification of global 5-methylcytosine (5-mC) levels in genomic DNA from leaves of 20-d-old *M. truncatula* plants grown *in vitro* by enzyme-linked immunosorbent assay-based immunoassay. For each DNA sample (*MtTdp1α*-depleted line Tdp1α-2a and control line CTRL, respectively), the percentage of 5-mC was quantified by plotting absorbance versus 5-mC percentage in the standard curve. For each *M. truncatula* line, duplicated DNA samples for two independent replications were analysed. Asterisks indicate statistical significance of differences determined using Student's *t*-test ($P < 0.05$).

Genome stability is an absolute requirement for plant survival under adverse environmental conditions and a main challenge for preserving high crop productivity in the context of global climate change. Physiology of DNA repair and transposon control deserves attention, being a source of information for crop genetic improvement as well as for designing novel breeding strategies.

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AUTHOR CONTRIBUTIONS

M.E.S. and M.D. performed most of the research and A.B. drafted the manuscript. P.L. carried out methylated cytosine analysis, A.M. and M.D. performed RNA-Seq and bioinformatics. M.C. and G.G. carried out *in vitro* work with *M. truncatula* lines. D.C., P.L. and A.B. revised the manuscript. M.D. and M.E.S. designed the experiment. A.B. supervised the study, and revised the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

File S1. Analysis of methylation patterns at LTR promoter regions

Figure S1. Changes in methylation patterns at LTR promoter regions detected using methylation-sensitive endonucleases combined with PCR-mediated amplification

(**A**) Distribution of LTR (Long Terminal Repeat) regions within the retrotransposon of the *Copia* and *Gypsy* plant superfamilies (modified from Pearce et al. 2000). gag, core particle components. Int, integrase. RT, reverse transcriptase. (**B**) The LTR region of the GYPSY-LTR_MT sequence (TCONS_00084148, Supplementary Table S2) selected in this study contains two *Hpa*II sites and a unique *Sac*I site. In case of cytosine methylation, the *Hpa*II and *Sac*I endonucleases are inhibited, thus the LTR region remains intact and the PCR-based amplification with the LTR-specific oligonucleotide primers (FW and REV) takes place. In case of demethylation, the *Hpa*II- and *Sac*I-mediated restriction will occur and no LTR template will be available for amplification. (**C**) Results of PCR amplification carried out on the *Hpa*II/*Sac*I-digested DNA extracted from Tdp1a-2a and CTRL lines, using the LTR-specific oligonucleotide primers. The expected amplification product (569 bp, spanning from nt 752 to nt 1321 of the LTR region) corresponding to cycles 25, 26, 27, 28 and 29 was visualized by agarose gel electrophoresis. M, molecular weight marker (GeneRuler^{TM} 50 bp DNA Ladder, MBI Fementas). (**D**) Densitometric analysis of the amplification product.

Table S1. List of gene sequences related to DNA transposons differentially expressed in the Tdp1α-2a line compared to CTRL Fold-Change represents the ratio of transcript values of the Tdp2α-2a line over the CTRL line

Each gene has been classified using the RepBase alignment tool(www.girinst.org/repbase/index.html). For each DNA transposons, the information currently available and references are indicated. hAT, Hatvine.IR, Inverted Repeats. Mad, Malus x domestica Borkh. MT, Medicago truncatula. TIRs, Terminal Inverted Repeats. TSDs, Target Site Duplications. VV, Vitis vinifera. (*) Sequences are accessible at http://www.ncbi.nlm.nih.gov/Traces/sra/sra.cgi under accession number SRP013555.

Table S2. List of LTR Retrotransposons (Gypsy, Gypsy-like, Copia) differentially expressed in the Tdp1α-2a line compared to CTRL

Retrotransposons are shown as groups. The Fold-Change represents the ratio of transcript values of the Tdp2α-2a line over the CTRL line. Each gene has been classified using the RepBase alignment tool (www.girinst.org/repbase/index.html). For each DNA transposons, the information currently available and references are indicated. LTR, Long Terminal Repeat. MT, Medicago truncatula. ORF, Open Reading Frame. PD, Phoenix dactylifera. VV, Vitis vinifera. (*) Sequences are accessible at http://www.ncbi.nlm.nih.gov/Traces/sra/sra.cgi under accession number SRP013555.

Table S3. List of LTR and NonLTR Retrotransposons differentially expressed in the Tdp1α-2a line compared to CTRL Retrotransposons are shown as groups

The Fold-Change represents the ratio of transcript values of the Tdp2α-2a line over the CTRL line. Each gene has been classified using the RepBase alignment tool (www.girinst.org/repbase/index.html). For each DNA transposons, the information currently available and references are indicated. LINE, Long Interspersed Element. LTR, Long Terminal Repeat. MT, Medicago truncatula. SINE, Short Interspersed Element. (*) Sequences are accessible at http://www.ncbi.nlm.nih.gov/Traces/sra/sra.cgi under accession number SRP013555.